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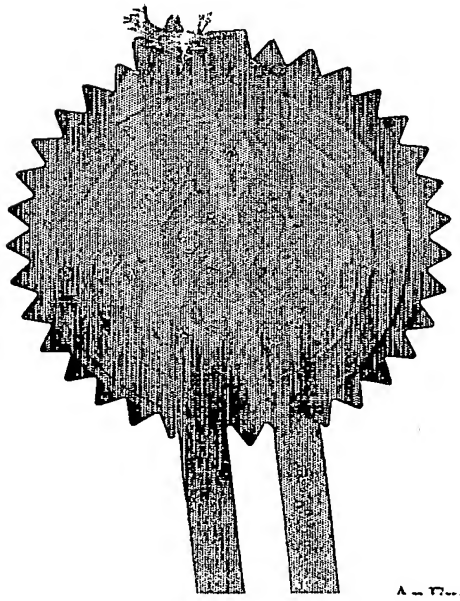

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1. Your reference — **P030741GB**

2. Patent application number *(The Patent Office will fill in this part)* — 0211904.8

24MAY02 E720943-1 D00019
P01/7700 0.00-0211904.8

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Medical Research Council
20 Park Crescent
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W1N 4AL

S8406240 01

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — **United Kingdom**

4. Title of the invention — **NUCLEAR TRANSFER**

5. Name of your agent *(if you have one)* — **Carpmaels & Ransford**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

43 Bloomsbury Square
London
WC1A 2RA

Patents ADP number *(if you know it)* — **83001**

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature Carpmaels & Ransford Date **23rd May 2002**

12. Name and daytime telephone number of person to contact in the United Kingdom

Carpmaels & Ransford

Huw Hallybone **020-7242 8692**

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20 Park Crescent
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If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

NUCLEAR TRANSFER

5. Name of your agent *(if you have one)*

Carpmaels & Ransford

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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NUCLEAR TRANSFER

The present invention relates to cloning procedures in which cell nuclei are transplanted into recipient cells. The nuclei are reprogrammed to direct the development of cloned embyros, which can then be transferred into recipient females to produce foetuses and offspring or used to produce embryonic cell lines.

All publications, patents and patent applications cited herein are incorporated in full by reference.

BACKGROUND

A fundamental question in cell and developmental biology concerns how nuclei progressively acquire differentiated functions. Although the nucleus of a fertilised egg is totipotent in that all of the differentiated cell types found in the adult organism can be derived from it, this is not the case for the vast majority of somatic nuclei in the adult animal. This limitation of the genomic potential of nuclei is progressively acquired during embryonic and post-embryonic development. Although in most cells the DNA sequence content of nuclei remains unchanged as development proceeds, the repertoire of genes that are expressed in a given cell type becomes limited. It also becomes more difficult to reactivate genes that are silenced in that cell type. This limitation is now known to reflect the imposition of epigenetic regulatory mechanisms on genes, especially through the assembly of stable repressive nucleoprotein complexes in the differentiated cell nucleus. The molecular mechanisms necessary to stably repress genes are gradually established as embryogenesis and post-embryonic development proceed. Remarkably, the egg and oocyte can reverse this process of repression, disassembling repressive features of nuclear organisation and, in particular circumstances, recreating a state, of pluripotency and even totipotency.

Covalent modifications to histone proteins have been proposed as the basis for an epigenetic code capable of extending the information potential of primary DNA sequences (*1*). This code could 'mark' the transcriptional status of genes and also provide a plausible self-templating mechanism to propagate chromatin status through DNA replication. Transcriptionally active euchromatin and inactive heterochromatin are characterized by generalized differences in histone modifications (*2*); for example, the global under-acetylation (particularly of H4) in heterochromatin domains such as those exemplified by the inactive X chromosome in mammals (*3*). In addition, acetylation and methylation at

specific residues, such as the reciprocal methylation of H3 at residues 4 and 9, are consistent features of active and inactive chromatin, respectively (*4, 5*).

A discussion of methylation at lysine 9 of H3 in animals may be found in Cowell *et al.* (2002) *Chromosoma*, 111:22-36. Contrary to the findings presented herein, Cowell *et al.* states that methylation at lysine 9 of H3 represents one of the most robust histone modifications and suggests that it is almost permanent in nature.

Although an increasing number of factors involved in transmitting gene expression have been identified we do not yet understood how, at a mechanistic level, transcriptional competence is conveyed to daughter cells. Polycomb (PcG) and Trithorax (TrxG) group proteins, appear to be crucial for the clonal inheritance of the inactive and active state of target genes, respectively, in diverse organisms (*6, 7*). In addition, some genes characterized as modifiers of position effect variegation (PEV) can also influence the transmission of epigenetic information (*8*). These modifiers may encode structural components of heterochromatin (such as HP1, allelic to *Suvar(2)5*), or enzymes that modify these components (such as the HMTase *Suv39h1*).

Interest in the basic molecular mechanisms involved in the imposition of epigenetic regulatory mechanisms on genes has been stimulated by the economic and medical implications of the cloning of animals by nuclear transfer from donor embryos and from adult cell nuclei. Unfortunately, the economic and medical exploitation of cloning technology has been hampered by the extremely low efficiency of cloning from adult cell nuclei with most clones dying during gestation.

Attempts to increase efficiency have included varying the source of donor nuclei. For instance, EP 930 009 describes the use of resting cells as nuclear donor cells whilst WO 99/53751 and Hoechedlinger and Jaenisch (2002) *Nature*, 415: 1035 to 1038 describes the use of lymphocytes as nuclear donors. However, Hoechedlinger and Jaenisch (2002) found that the use of lymphocytes as nuclear donor cells was relatively inefficient and concluded that the efficiency was about ten times lower than that from other donor cell populations. It was suggested that the low efficiency could be due to inefficient reprogramming of the lymphocyte genome or differences in the sensitivity of the lymphocyte nuclei to the nuclear transfer protocol.

In view of the foregoing, it will be appreciated that there is a need for an improved understanding of the mechanisms underlying epigenetic regulation and a need for new approaches towards improving the efficiency and success of nuclear transfer procedures.

THE INVENTION

The present invention is based on the discovery that cells which have histone hypomethylation may advantageously be used as nuclear donor cells. By using cells which have histone hypomethylation the efficiency of nuclear transfer may be increased.

A first aspect of the invention provides a method of producing an animal embryo, the method comprising transferring from a nuclear donor cell which has been selected on the basis that it is histone hypomethylated at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development into a suitable recipient cell.

The term "embryo" as used herein includes all concepts of an animal embryo such as an oocyte, egg, zygote or an early embryo. More specifically, the term "embryo" used herein includes morulas (8-16 cells), morulas (16-32 cells) and blastocysts (64 cells and above).

The term "nuclear donor cell" as used herein includes a cell from which at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development is transferred into a suitable recipient cell. Similar expressions e.g. "nuclear transfer" should be interpreted in a likewise manner.

By a "cell which has been selected on the basis that it is histone hypomethylated" we include:

(i) testing a cell to determine if it is histone hypomethylated and selecting the cell if it is found to be histone hypomethylated;

(ii) experimentally determining that a first cell is histone hypomethylated and selecting a second cell (the nuclear donor cell) which is similar or identical to the first cell to thereby select a histone hypomethylated cell to be used as a nuclear donor cell;

(iii) selecting a histone hypomethylated cell by selecting a cell of a type which has been previously determined as being histone hypomethylated (e.g. a resting B lymphocyte) or which has been previously determined as being likely to be histone hypomethylated.

Preferably technique (ii) or (iii) is used.

With respect to technique (ii), by the second cell being "similar" to the first cell we refer to the second cell being sufficiently similar to the first cell (i.e. having a sufficient number of characteristics in common) such that it is reasonable to infer that because the first cell exhibits histone hypomethylation the second cell also exhibits histone hypomethylation. Preferably, the first and second cells are from the same population of cells.

With respect to technique (iii), by a type of cell which has been previously determined as being likely to be histone hypomethylated we include a type of cell of which at least 70%, 80%, 90%, 95%, 99% or 99.5% of cells of that type are hypomethylated.

Histone hypomethylated cells may be readily distinguished from methylated cells due to the considerable and readily-observable differences in their respective levels of histone methylation. Indeed, the level of methylation in hypomethylated cells appears to be negligible or absent (or at least undetectable). Thus, for practical purposes methylation may generally be considered to be an all or nothing (or almost nothing) event. Accordingly, the skilled person will readily be able to appreciate whether a cell is hypomethylated or not.

Preferably, a cell is regarded as being histone hypomethylated if histone methylation is negligible or absent (absent being used herein to mean undetectable).

As demonstrated in the Example below, cells which are hypomethylated include G_0 (resting) lymphocytes and some liver cells (as indicated below these liver cells may be Kupfer cells). Also, the paternal genome, early after fertilisation has been identified as being hypomethylated (see Cowell *et al.* (2002)).

Cells which are not hypomethylated include activated lymphocytes, serum starved fibroblasts and some post-mitotic cells (e.g. cumulus cells and multinucleated muscle fibres).

In one embodiment, a cell is regarded as being histone hypomethylated if it has 10% or less (and more preferably $\leq$ 8%, 5% or 2%) of the level of histone methylation of one or more (and preferably any) of the cell types listed above as examples of cells which are not hypomethylated. Preferably, a cell is regarded as being histone hypomethylated if it has 10% or less (and more preferably $\leq$ 8%, 5% or 2%) of the level of histone methylation of an activated lymphocyte.

Methods of quantifying histone methylation will be known to those skilled in the art or can be readily devised by those skilled in the art. For example, a semi-quantitative western blotting approach may be used.

Histone methylation may be assessed in various ways as outlined below.

Histone methylation may be assessed with regard to one or more histone types. Preferably, the level of histone methylation is assessed with regard to H3 and/or H4, preferably with regard to H3.

The assessment of histone methylation may involve assessing whether one or more histone residues are methylated. Obviously, for the assay to be meaningful only methylation at histone residues which are known to undergo histone methylation is assessed.

With regard to techniques (ii) and (iii), in one embodiment it is preferred that the level of histone methylation of said first cell or of said cell type is assessed on the basis of methylation at one or more residues of H3.

Histone residues which may be methylated include lysine and arginine residues. In one embodiment, methylation at one or more lysine residues is assessed. In another embodiment methylation at one or more arginine residues is assessed. Preferably, methylation at one or more lysine residues and at one or more arginine residue is assessed.

Lysine residues of H3 which may be methylated in mammals include residues 4, 9, 27 and 36 (Rice and Allia (2001) *Current Opinion in Cell Biology*, 13:263-273; and Richards and Elgin (2002) *Cell* 108, 489-500). Preferably, methylation at one, two, three or four of these lysine residues is assessed. Preferably, methylation of $H3^{K4}$ or $H3^{K9}$ is assessed. Preferably, methylation of both $H3^{K4}$ and $H3^{K9}$ is assessed.

Preferably, methylation at ≥ two, three, four, five or six histone residues is assayed.

The assessment of histone methylation may involve assessing the extent of methylation (ie. mono-, di- or tri-methylation) at one or more residue(s).

It will be appreciated that an assessment of histone methylation may involve assaying different histones for methylation and/or different histone residues for methylation and/or the extent of methylation at different residues.

Preferably, a cell which is regarded as being histone hypomethylated has negligible or absent (i.e. undetectable) methylation at ≥ one, two, three, four, five or six histone residues.

Antibodies may be used to assess histone methylation and techniques for raising antibodies with desired specificities will be well known to those skilled in the art. Moreover, some antibodies with appropriate specificities are commercially available (see the Materials and Methods section below).

To assess histone methylation, immunofluorescence-based approaches or protein-based technologies (ie. cells lysates and western blotting) may be used (see the Examples section below).

Preferably, the nuclear donor cell employed in the present invention is a mammalian cell. Preferably, the recipient cell is a mammalian cell. Preferably, the nuclear donor cell and the recipient cell are both mammalian cells; preferably they are both ungulate, rat or murine cells.

In an alternative embodiment the nuclear donor cell and/or recipient cell is not a mammalian cell. The nuclear donor cell and/or recipient cell may, for example, be a *Xenopus* cell.

Preferably, donor cells and recipient cells from the same species are used. Preferably, the donor cell and recipient cell are both human cells or mouse cells.

Cells derived from populations grown *in vivo* or *in vitro* and containing 2n chromosomes (e.g. those in G0 or G1) or greater than 2n chromosomes (e.g., those in G2, which are normally 4n) may act as nuclear donor cells.

An example of an *in vivo* source of the 2n donor nucleus is a cumulus cell. One embodiment of the invention contemplates using donor nuclei taken from either *in vivo* or *in vitro* (i.e., cultured) sources of 2n adult somatic cells including, without limitation, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B or T lymphocytes, macrophages, monocytes, nucleated erythrocytes, fibroblasts, Sertoli cells, cardiac muscle cells, skeletal muscle cells, smooth muscle cells, and other cells from organs including, without limitation, skin, lung, pancreas, liver, kidney, urinary bladder, stomach, intestine, bone, and the like, and their progenitor cells where appropriate.

In one embodiment, the donor cell is a resting cell (G_0), preferably a resting B lymphocyte.

In another embodiment of the invention, the donor adult somatic cell is "2-4C"; that is, it contains one to two times the diploid genomic content, as a result of replication during S phase of the cell cycle. This donor cell may be obtained from an *in vivo* or an *in vitro* source of actively dividing cells including, but not limited to, epithelial cells, hematopoietic cells, epidermal cells, keratinocytes, fibroblasts, and the like, and their progenitor cells where appropriate.

In one embodiment of the invention it is preferred that the donor cell is not selected from the group consisting of: a resting lymphocyte, a resting B lymphocyte, a liver cell, or a Kupfer cell.

Optionally the donor nucleus may be genetically modified. The donor nucleus may contain one or more transgenes and the genetic modification may take place prior to nuclear transfer and embryo reconstitution. Such a genetically modified donor nucleus may be used in the creation of a transgenic animal.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose germ line an exogenous DNA sequence has been added.

Preferably, the recipient cell is a one cell zygote, enucleated oocyte, embryonic stem (ES) cell or any other type of cell which may facilitate in the reprogramming of the donor nucleus. As will be appreciated from below, the recipient cell may be the "ultimate" recipient cell in which case the resulting embryo may directly give rise to a foetus or animal (offspring). Alternatively, in the case of serial nuclear transfer (discussed below), the recipient cell may not be the "ultimate" recipient cell and it may act as a nuclear donor cell.

Preferably, the enucleated oocyte is a mammalian enucleated oocyte. Enucleation may be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate

(depending on the recipient cell), or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

Oocytes that may be used in the method of the invention include both immature (e.g., GV stage) and mature (i.e., Met II stage) oocytes. Mature oocytes may be obtained, for example, by inducing an animal to super-ovulate by injections of gonadotrophic or other hormones (for example, sequential administration of equine and human chorionic gonadotrophins) and surgical harvesting of ova shortly after ovulation (e.g., 80-84 hours after the onset of estrous in the domestic cat, 72-96 hours after the onset of estrous in the cow and 13-15 hours after the onset of estrous in the mouse). Where it is only possible to obtain immature oocytes, they are cultured in a maturation-promoting medium until they have progressed to Met II; this is known as in vitro maturation ("IVM"). Methods for IVM of immature bovine oocytes are described in WO 98/07841, and for immature mouse oocytes in Eppig & Telfer (Methods in Enzymology 225, 77-84, Academic Press, 1993).

Preferably, the recipient cell to which the donor cell nucleus is transferred is an enucleated metaphase II oocyte, an enucleated unactivated oocyte or an enucleated preactivated oocyte. At least where the recipient is an enucleated metaphase II oocyte, activation may take place at the time of transfer. Alternatively, at least where the recipient is an enucleated unactivated metaphase II oocyte, activation may take place subsequently.

Once suitable donor and recipient cells have been selected, it is necessary for the nuclear material of the former to be transferred to the latter. The nuclear donor cell can be transferred intact into a suitable recipient cell, optionally with a broken cell membrane. Alternatively, the nuclear contents of the donor cell (or a portion of the nuclear contents including at least the minimum chromosomal material able to support development) can be directly inserted into the cytoplasm of an enucleated oocyte.

Conveniently, nuclear transfer is effected by fusion. Three established methods which have been used to induce fusion are: (i) exposure of cells to fusion-promoting chemicals, such as polyethylene glycol; (ii) the use of inactivated virus, such as Sendai virus; and (iii) the use of electrical stimulation.

Alternatively, nuclear transfer is effected by microinjection.

Before or (preferably) after nuclear transfer (or, in some instances at least, concomitantly with it), it is generally necessary to stimulate the recipient cell into development by parthenogenetic activation, at least if the cell is an oocyte. In one embodiment, the

activation step takes place from zero to about six hours after nuclear transfer in order to allow the nucleus to be in contact with the cytoplasm of the oocyte for a period of time prior to activation of the oocyte. Activation may be achieved by various means which will be well known to those skilled in the art.

There are several options for which the embryos made by the present invention may be used for.

In one embodiment, the embryo may be used in serial nuclear transfer. Thus, a second aspect of the invention provides a method of producing an animal embryo, the method comprising transferring from a nuclear donor cell at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development into a suitable recipient cell wherein the nuclear donor cell is obtained from an embryo obtained by the method of the first aspect of the invention.

Preferably, the nuclear donor cell obtained from an embryo obtained in accordance with the first aspect of the invention has been selected on the basis that it is histone hypomethylated.

It will be appreciated that the embryo obtained by the method of the second aspect of the invention may be used for further rounds of serial nuclear transfer.

Preferably, an embryo obtained by the first or second aspects of the invention is allowed to develop into a foetus or animal (i.e. live offspring). Thus, a third aspect of the present invention provides a method of producing a foetus the method comprising allowing an embryo obtained by the first or second aspect of the invention to develop into a foetus.

The step of allowing the embryo to develop may include the substep of transferring the embryo to a female mammalian surrogate recipient, wherein the embryo develops into a viable foetus. The embryo may be transferred at any stage, including from the two-cell to morula/blastocyst stage, as known to those skilled in the art.

A fourth aspect of the invention provides a method of producing a non-human animal the method comprising allowing an embryo obtained by the first or second aspects of the invention or a foetus obtained by the third aspect of the invention to develop into said non-human animal.

Those skilled in the art will appreciate that the cloned embryos of the present invention may be combined with fertilized embryos to produce chimeric embryos, foetuses and/or

offspring. Such chimeric embryos, foetuses and/or offspring are also included within the scope of the present invention.

In another aspect of the invention an embryo of the present invention is used in the preparation of an embryonic stem cell line. Thus, a fifth aspect of the present invention provides a method of producing an embryonic stem cell line, the method comprising transferring an embryo obtained by the method of the first or second aspect of the invention to a culture system.

A sixth aspect of the invention provides a method of producing an embryonic stem cell line, the method comprising isolating the inner cell mass of an embryo obtained by the method of the first or second aspect of the invention and transferring the inner cell mass to a culture system.

An embryonic cell line could find beneficial application in its use to generate embryonic stem cells from a patient as a source of compatible undifferentiated cells to be used in transplantation for the therapy of degenerative diseases.

In an seventh aspect of the invention, a cell could be treated to artificially reduce the level of histone methylation so as to render the cell histone hypomethylated. The cell could be employed as a nuclear donor cell in the above described methods of the present invention. The treatment may be chemical or enzymatic and may, for example, involve treatment with a histone demethylase or with a histone methyltransferase (HMT) inhibitor.

An eighth aspect of the invention relates to the embryos, foetuses, non-human animals, and embryonic cells obtained by the methods described above.

A ninth aspect of the invention relates to the use of histone hypomethylation status as an indicator of the suitability of a cell to act as a nuclear donor cell. Histone hypomethylation status may be assessed as described above.

A tenth aspect of the invention provides a method of selecting a cell to be used as a nuclear donor cell the method comprising selecting said cell on the basis that it is histone hypomethylated.

In principle, the invention is applicable to all animals, including birds, such as domestic fowl, amphibian species and fish species. In practice, however, it will generally be to placental mammals that the greatest commercially useful applicability is presently envisaged. It is with ungulates, particularly economically important ungulates such as

cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful, both as a means for cloning animals and as a means for generating transgenic or genetically modified animals. It should also be noted that the invention is also likely to be applicable to other economically important animal species such as, for example, horses, llamas or rodents e.g. rats, mice, rabbits and humans. However, due to ethical considerations, it may be desirable for certain aspects of the invention not to be applied to humans.

The present invention will now be described by reference to the accompanying Examples which are provided for the purposes of illustration and are not to be construed as being limiting on the present invention.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1: Kinetics of CD69 expression and Brd U incorporation in quiescent mouse B lymphocytes stimulated with anti-1gM and anti CD40 antibodies in the presence of IL-4.

Figure 2: HP1 (M31) and Ikaros proteins are upregulated and redistributed to constitute heterochromation domains in B lymphocytes following mitotic stimulation

Figure 3(a): Time course of Ikaros, HP1, Ezh2 and Bmi-1 protein upregulation in nuclear extracts prepared from quiescent mouse B lymphocytes and following mitotic stimulation.

Figure 3(b): Pc-G proteins EzHz and Bmi-1 are upregulated and redistributed upon lymphocyte activation.

Figure 4: Evidence that methylated $H3^{K9}$ levels are low or absent in quiescent lymphocytes, but reinstated upon entry into the cell cycle.

Figure 5: Evidence that quiescent (G_0) mouse B lymphocytes lack histone $H3^{K9}$ and $H3^{K4}$ methylation as judged by immunofluorecence (a) and Western blotting analysis (b).

Figure 6: (supplementary information (i)): Specificity of 4xmethylH3-K9 and methyl H3-K9 antibodies for di and tri-methylated H3-K9

Figure 7 (supplementary information (ii)): $H3^{K9}$ is absent from a population of G_0 cells in the adult mouse liver. The hypomethylated cells may be Kupfer cells.

EXAMPLE

Materials and Methods

Purification and stimulation and characterisation of B cells

Resting B cell Purification from Spleen

Normal resting B cells were prepared from the spleens of young (6 - 10 week old) mice using a magnetic immuno-depletion approach with reagents provided commercially from Miltenyi Biotech. Briefly, individual spleens were dissected and minced to yield single cell suspensions, treated with Geyes solution to lyse erythrocytes (see below), washed and incubated with anti-CD43 (Ly-48)-coupled micro beads (Miltenyi Biotech) according to manufacturers' instructions. CD43 positive cells (T cells, plasma B cells and granulocytes) were removed by magnetic separation and untouched B cells isolated.

Lysis of Erythrocytes Using Geyes Solution

Geyes solution was prepared by mixing 20 parts stock solution A (650mM NH_4Cl, 25mM KCl, 4mM $Na_2HPO_4.12H_2O$, 1mM KH_2PO_4, 28mM Glucose) to 5 parts stock solution B (20mM $MgCl_2.6H_2O$, 6mM $MgSO_4.7H_20$, 30mM $CaCl_2$) to 5 parts stock solution C (267mM $NaHCO_3$) to 70 parts sterile distilled water. To lyse erythrocytes cells were re-supended in 1ml of medium per spleen, 4ml Geyes solution added and then held on ice for 2 min before washing in media.

B cell Activation

B cell activation was induced by culturing cells in media (IMDM containing 10% FCS (Sigma batch no. 69H3357 and antibiotics) containing 20 µg/ml purified anti-CD40 (monoclonal antibody FGK45), 10µg/ml purified anti IgMµ (monoclonal antibody H3074) and 2% IL-4 containing supernatant (from a T-helper cell line).

Cells were phenotyped using fluorecently labelled antibodies against B220 and CD69 (BD Pharmingen).

Incorporation of BrdU into B Cells

Ex vivo resting mature B cells were isolated and cultured in media containing 50µM BrdU with either IL-4 (to preserve resting status) for 24 hrs, or activated with anti-IgM, anti-CD40 and IL-4 as above. Immunostaining of incorporated BrdU was as previously described (Huesmann et al., 1991). Briefly at the appropriate time point cells were fixed in ice cold 70% EtOH and stored at 4°C. To examine BrdU incorporation cells were washed in ice cold PBS, denatured in

3M HCl with 0.5% Tween for 20min, followed by 0.01M sodium tetraborate solution for 3min. Then the cells are washed 2 x in ice cold PBS and the incubated in FITC conjugated anti-BrdU monoclonal antibody (BD Pharmingen) before been washed and analysed by FACScan (Becton Dickenson).

Immunofluorescence and antibodies

Antibodies Used

Antisera used for immunofluorescence and western studies were; anti N- and C- terminus Ikaros (Hahm et al., 1998), anti M31 (Serotec) (Wreggett et al., 1994), anti lamin B (Santa Cruz), human CREST autoimmune sera, anti-4x methyl$H3^{K9}$ (Peters et al., 2001), anti 1x methyl $H3^{K9}$, anti methyl $H3^{K4}$, anti acetyl $H3^{K9}$, anti acetyl $H3^{K14}$ (all from Upstate Biotechnologies), anti pan-acetyl H4 (Serotec), anti Enx1 (Sewalt et al., 1998), anti EED (van der Vlag and Otte, 1999), anti BMI1 (Gunster et al., 1997), anti YY1 (Santa Cruz). Other antibodies used were anti HDAC2 (Santa Cruz), anti ORC1 (Serotec) and anti PCNA (Sigma).

Immunofluorescence Staining of Nuclear Proteins

Cells were attached to glass coverslips coated in poly-L-lysine and washed in PBS. The cells were fixed in 2% paraformaldehyde for 10 minutes, washed in PBS and then quenched with 0.05M NH_4Cl in PBS for 5 minutes, before further washing in PBS and permeabilisation with 0.3% Triton in PBS for 5 minutes. The preparations were then sequentially incubated in blocking solution (0.2% Fish gelatin (Sigma) in PBS) for 30 minutes and then in the appropriate primary antisera (diluted in blocking buffer with 5% NGS), for 60 minutes in a humid chamber. After three consecutive three-minute rinses in blocking solution, cells were incubated for a further 30 minutes with an appropriate fluorochrome-labelled secondary antibody diluted appropriately in blocking buffer and 5% NGS. Slides were washed twice (3 minutes/wash) in wash buffer, once in PBS alone and mounted in Vectashield (Vector) supplemented with DAPI (0.1 µg/ml). In situations where goat primary antibodies were utilised FCS was used in the block instead of NGS at the same concentration.

Immunofluorescence Staining of Histone Modifications

Immunofluorescence staining of histone modifications were essentially performed as described by Peters *et al* (2001) with minor modifications. Cells were attached to glass coverslips coated in poly-L-lysine and washed in PBS. The cells were then fixed in 2%

paraformaldehyde for 10 minutes, washed in PBS and then incubated in wash buffer for 5 minutes (wash buffer; PBS, 0.2% BSA, 0.1% Tween20). Preparations were then sequentially incubated, in a humid chamber, in blocking solution (blocking solution; PBS, 10% normal goat serum, 2.5% BSA, 0.1% Tween20) for 30 minutes, primary antibody diluted in blocking solution for 1 hour and Alexa 488 conjugated goat anti rabbit secondary antibody diluted in blocking buffer. Samples were then mounted in Vectashield (Vector) supplemented with DAPI (0.1 μg/ml).

Preparation of Nuclear Extracts

Preparation of Nuclear Fractions from *Ex Vivo* B cells

B cells were washed in ice cold PBS, centrifuged at 600g for 4 minutes in a chilled centrifuge (4°C) and resuspended in ice cold nuclei lysis buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, supplemented with protease inhibitor cocktail and phosphatase cocktail (Sigma) and 1 mM DTT), nuclei lysis buffer containing 0.75% NP40 was then added dropwise until the concentration of NP40 reached 0.15% and then left on ice for 2 minutes and then centrifuged at 400g for 2 minutes. The non-chromatin fraction supernatent was then collected. Nuclei washed once in lysis buffer and centrifuged again as previously. Chromatin was solubilised by DNA digestion with 1 mg/ml of RNAse-free DNAse I (Sigma) in lysis buffer 30 minutes at 30°C. Then $NH_4(SO_4)$ was added from a 1 M stock solution in lysis buffer to a final concentration of 0.25 M. After 5 min on ice, samples were pelleted again by centrifuging at 1500g for 3 minutes and the DNAse I soluble material collected. The pellet of DNAse I insoluble material was then solubilised in Urea buffer (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl pH 8.0). The protein extracts were quantified and then stored at -70°C.

Acid Extraction of B cells

Histone proteins were isolated from whole cells by acid extraction. $1x10^7$ B cells were pelleted and resuspended in 1 ml PBS (4°C) and centrifuged (500g for 5 minutes) and the supernatent removed. Cell pellet was then resuspended in 180μl of ice cold lysis buffer (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 1.5mM PMSF), 20μl of 2M HCl added and then incubated on ice for 30 minutes. Following acid lysis the solution was centrifuged 11000g for 10 min at 4°C and the supernatent of acid soluble proteins collected. The solution was then sequentially dialysed against 0.1M acetic acid,

twice for 1 hour, and then against H_2O for 1 hour, 3 hours and overnight respectively. The protein solution was quantified and then stored at -70°C.

Results

In this study, we assessed the contribution of histone modifications to cellular memory using purified resting (G_0) B lymphocytes. These cells can be stimulated to enter the cell cycle and give rise to progeny in which the correct lineage affiliation and developmental-stage is transmitted. Non-cycling, $B220^+$ B lymphocytes were isolated from the spleens of normal mice (by CD43 depletion (*9*)) and stimulated with anti-IgM, anti-CD40 antibodies in the presence of interleukin-4 (IL-4). Under these conditions the cells were induced to express the activation marker CD69 within 24 hours. DNA synthesis, as detected by BrdU incorporation occurred later, 48 to 72 hours after stimulation (figure 1). The distribution of heterochromatin-associated proteins (Ikaros, HP1β and CENP-A) in quiescent cells and after mitotic stimulation, was monitored by immuofluorescence (IF) and visualised by confocal microscopy (figure 2). In these studies all microscope settings and the laser power were kept constant so that the relative abundance and distribution of proteins could be compared directly. In purified resting B populations, Ikaros protein was low or absent but increased following activation and re-located to centomeric domains as reported previously (*10*). Low levels of HP1β/M31 were detected in the nuclei of resting B cells, which increased slightly over 24 hours, but did not coincide with DAPI-intense centromere clusters (figure 2) until 48-72 hours after activation. At this time, as lymphocytes began active cell division (figure 1), HP1β and Ikaros proteins co-localised around centromeric DNA as previously reported (*11*).The kinetic re-distribution Ikaros and HP1β/M31 was confirmed using antibodies specific for alternative regions of these proteins (not shown). This, together with the demonstration that CREST antisera detected centromeres throughout B cell activation (figure 2, lower panels) rules out that technical problems (such as epitope masking or restricted antibody accessibility) were responsible for HP-1β/M31 not being localized to constitutive heterochromatin in G_0 cells.

The protein composition of chromatin in resting and activated B cells was further investigated using western blotting. Nuclei were isolated from resting and activated cells by partial NP40 lysis; a treatment that results in the removal of non chromatin-bound proteins. Samples were then subjected to DNase I digestion and soluble (NE-s) or insoluble (NE-i) nuclear extracts were derived and analyzed by SDS-PAGE and western blotting

(figure 3a). Controls for this analysis included PCNA, a component of the DNA replication machinery synthesized as cells enter S-phase and ORC1, a protein which marks origins of replication in quiescent and cycling cells (used here to estimate the equivalence of protein loading). Low levels of PCNA were detected 48 hours after stimulation but this protein became abundant in chromatin fractions after 72 hours. This observation is consistent with most lymphocytes entering S-phase at this time, confirming the results of BrdU incorporation data (figure 1) and previous analyses (*10*). Ikaros proteins corresponding to the major isoforms present in lymphocytes (isoforms I, II, (*12*)), although absent from G_o samples (0 hours), were seen to accumulate in NE-i fractions 48-72 hours after stimulation. M31 protein was present in the soluble chromatin compartment (NE-s) throughout B cell activation but showed a progressive recruitment to insoluble fractions (NS-i) with activation. This data is consistent IF analysis (figure 2) showing that M31 protein present within quiescent B cells is up-regulated and redistributed to condensed chromatin domains as cells begin division. These results suggest that the composition of heterochromatin in lymphocytes may vary according to the activation status of the cell.

We extended this analysis to the distribution of PcG proteins in resting and activated B lymphocytes (figures 3a and 3b). Pc-G proteins are thought to function as large multi-component complexes that have some similarities with heterochromatin (*13, 14*) but are targeted principally to euchromatin. Four PcG proteins were studied in detail; Ezh2, Eed, Bmi1 and YY1. We observed that Ezh2 and Bmi-1 proteins were selectively up-regulated during B cells activation. Both proteins were detected in chromatin-bound and soluble nuclear fractions and their abundance increased following lymphocyte activation. In contrast, Eed levels (detected by an antibody that recognizes both putative proteins encoded by two alternatively transcribed mRNAs) (*15*) remain relatively unchanged in resting and activated B lymphocytes. Similarly, YY1 was expressed equivalently in both populations, although this protein was seen predominantly within the non chromatin-bound and cytoplasmic fractions.

The selective up-regulation of Ezh2 and Bmi-1 PcG proteins in activated B lymphocytes was confirmed by IF labeling (figure 3b). Ezh2 staining was extremely low in resting cells and increased to a high level of nuclear staining in actively proliferating cells (72 hours, top panel). Small nuclear foci of Bmi-1 were evident in some resting B cells and the intensity and number of nuclear foci increased dramatically upon cell activation. This contrasted with the broadly equivalent distribution of Eed protein and YY1 protein seen in

resting and activated cells. In addition, YY1 was detected both within the nucleus and cytoplasm (Figure 3b), consistent the reported cytoplasmic location of this protein in other cell types (*16-18*) and the presence of YY1 in cytoplasmic fractions of B- lymphocytes (shown in figure 3a).

Global changes in chromatin components may parallel the reported nuclear redistribution of genes that occurs in response to cellular activation of quiescent lymphocytes and fibroblasts (Brown, Bickmore etc). HP1 binding to heterochromatin is known to be regulated by the histone methyl transferase Suv39h1 which catalyses the methylation of H3 at lysine 9 ($H3^{K9}$) and provides a high affinity binding site for M31 (*19*). We therefore asked whether M31 relocation in activated lymphocytes was indicative of underlying changes in histone methylation. In addition, since Ikaros and HP1β/M31 proteins are recruited to the same nuclear compartments (*10*) with apparently similar kinetics, we asked whether Ikaros recruitment was dependent on chromatin-bound HP-1. To assess these questions, B lymphocytes from normal mice and mice lacking *Suv39h1 and Suv39h2* (*Suv39h-/-*) were analyzed by IF and Western blotting using antibody raised against a branched peptide containing four dimethylated H3K9 termini 4x(methylH3K9). This "branched methyl" antibody detects di- and trimethylated $H3^{K9}$ (see supplementary information) and labels both centromeric heterochromatin and euchromatic sites (*20*). *Suv39h-/-* deficient mice lack M31 enrichment at the constitutive heterochromatin (*19, 20*). Unexpectedly, methylated $H3^{K9}$ could not be detected in quiescent lymphocytes from either normal (wild type) and *Suv39h -/-* mice (figure 4). However, following activation for 72 hours, methylated $H3^{K9}$ was abundant within the nucleus of lymphocytes derived from normal and *Suv39h -/-* mice. In wild type cells labeling was significantly enriched around the DAPI dense regions, consistent with previous reports (*20*). In activated lymphocytes from *Suv39h -/-* male or female mice $H3^{K9}$ labeling was not restricted to, or enriched at, DAPI-intense regions (consistent with (*20*)). In male cells, this staining appeared evenly distributed throughout the nucleoplasm, whereas in female cells enrichement was seen at a single DAPI-dense region corresponding to the Barr body.

To examine whether Ikaros proteins bind to centromeric heterochromatin in the absence of local M31 accumulation, Ikaros distribution was analyzed in activated *Suv39h-/-* lymphocytes. Ikaros proteins were not detected in resting B cells from wild type or *Suv39h-/-* mice (not shown), but following activation (72 hours), similar amounts of Ikaros protein were detected in both samples. Moreover, in both wild type and *Suv39h-/-* cells

Ikaros protein localized to DAPI-intense areas of centromeric heterochromatin (figure 4, lower panels). This shows that Ikaros localisation at centromeric heterochromatin is independent of HP1 recruitment and is compatible with findings that HP1 interacts with histone protein (*19, 21*) whereas Ikaros directly binds to repetitive DNA sequences that flank centromeres (*22*). These data also indicate that the high level of methylated $H3^{K9}$ present at constitutive heterochromatin, faculative heterochromatin (the inactive X-chromosome) and within euchromatin, are not constitutive in B cells but acquired upon entry into cell cycle.

To assess whether mitotic stimulation results in additional modifications to histones, IF and western blots were performed using reagents specific for acetylated $H3^{K9}$, $H3^{K14}$, H4, and methylated $H3^{K4}$ (figure 5a and 5b). The dramatic difference in the abundance of methylated $H3^{K9}$ in cycling and non-cycling lymphocytes was verified using a second methylH3K9-specific antibody raised against a single (non-branched) dimethylated $H3^{K9}$ peptide. This antibody, like the 4xmethyl H3-K9 antibody, showed hardly any staining of resting B cells (0 hours), but in activated B lymphocytes (72 hours) labeling was substantially increased. As indicated by a previous report (*20*) the 'methyl H3-K9' antibody did not preferentially locate to DAPI-bright heterochromatin regions. To ask whether enhanced H3 methylation was also induced at other residues, we examined the extent of H3 methylation of lysine residue 4. $H3^{K4}$ methylation has been correlated with gene activity in other systems (*4, 5, 23*). In quiescent B lymphocytes $H3^{K4}$ methylation was low or absent. However, high levels were induced by mitotic stimulation and evident diffusely throughout the nucleus. To address the possibility that histone methylation remained undetected in G_0 populations because of an unusual packaging of histones in resting cells, lysates prepared from resting and activated B lymphocytes were analyzed by western blotting. The extent of histone acetylation in cycling and non-cycling lymphocytes was also compared for reference. As shown in figure 5b, only low levels of methylated $H3^{K9}$ and $H3^{K4}$ were detectable in lysates prepared from resting B lymphocytes. In samples prepared from activated B lymphocytes, methylated $H3^{K9}$ and $H3^{K4}$ was increased, consistent with results obtained by IF. Blotting with a control antibody that detects unmodified amino-terminal H3 confirmed that similar amounts of H3 histones were present in each of these samples. These data confirmed that mitotic stimulation of quiescent B lymphocytes induced a global increase in H3 methylation. Interestingly, $H3^{K9}$ and $H3^{K4}$ methylation was evident within 24 hours of activation (figure 5b), implying that

histone methyl transferases, such as Suv39h1, are active in cells prior to the onset of DNA replication. In contrast to these findings, global acetylation of $H3^{K9}$, $H3^{K14}$ and H4 appeared remarkably similar in resting and activated B lymphocyte populations. This is illustrated in the images shown in the lower panels of figure 5a, in which acetylated $H3^{K9}$, $H3^{K14}$ and H4 were readily detected in resting lymphocytes. These data suggest that histone acetylation is robustly retained by quiescent cells whereas histone methylation appears to be a relatively unstable epigenetic trait. One explanation for these results is that basal transcription of genes in G_0 lymphocytes is sufficient to maintain acetylation of the lymphocyte genome. Histone methylation, in contrast, may only be required only when overall gene activity is increased (following mitotic stimulation) to selectively mark the epigenetic status of a gene prior to DNA synthesis. A second possibility, that lack of histone methylation is peculiar to non-cycling lymphocytes, is ruled out by the observation that in liver approximately half of all cells lack $H3^{K9}$ methylation (see supplementary information). In other resting cell populations such as serum starved fibroblasts and some post-mitotic cells (cumulus cells and multinucleated muscle fibres), we observed that $H3^{K9}$ methylation was retained but not focussed at constitutive heterochromatin (JB unpublished). These differences in the extent of $H3^{K9}$ methylation in quiescent fibroblasts, lymphocytes and liver cells may reflect the extent of 'dormancy' of these populations indicated by the time delay before cells resume cycling. In any case, the surprising demonstration that histone methylation is dynamically up-regulated in resting lymphocytes prior to DNA replication, suggests that this modification is not a 'static' epigenetic imprint but instead is likely to be important in transmitting epigenetic information through cell division.

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CLAIMS

1. A method of producing an animal embryo, the method comprising transferring from a nuclear donor cell which has been selected on the basis that it is histone hypomethylated at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development into a suitable recipient cell.

2. The method of claim 1 wherein the nuclear donor cell has been selected by experimentally determining that a first cell is histone hypomethylated and selecting a second cell which is similar or identical to the first cell to thereby select a histone hypomethylated cell to be used as said nuclear donor cell.

3. The method of claim 2 wherein said first cell and second cell are from the same population of cells.

4. The method of claim 1 wherein the nuclear donor cell has been selected by selecting a cell of a type which has been previously determined as being histone hypomethylated or which has been previously determined as being likely to be histone hypomethylated.

5. The method of any one of claims 2 to 4 wherein the level of histone methylation of said first cell or of said cell type when histone hypomethylated is negligible or absent.

6. The method of any one of claims 2 to 5 wherein the level of histone methylation of said first cell or of said cell type when histone hypomethylated is assessed on the basis of methylation at one or more residues of H3.

7. The method of any one of claims 2 to 6 wherein the level of histone methylation of said first cell or of said cell type when histone hypomethylated is assessed on the basis of methylation at one or more lysine residues.

8. The method according to claim 7 wherein the level of histone methylation is assessed on the basis of methylation at one, two, three or four of the following lysine residues: residues $H3^{K4}$, $H3^{K9}$, $H3^{K27}$ and $H3^{K36}$.

9. The method according to claim 8 wherein the level of histone methylation is assessed on the basis of methylation at $H3^{K4}$ and $H3^{K9}$.

10. The method according to any one of the preceding claims wherein the nuclear donor cell is a mammalian cell.

11. The method according to any one of the preceding claims wherein the recipient cell is a mammalian cell.

12. The method according to any one of the preceding claims wherein the recipient cell is an enucleated oocyte.

13. A method of producing an animal embryo, the method comprising transferring from a nuclear donor cell at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development into a suitable recipient cell wherein the nuclear donor cell is obtained from an embryo obtained by the method of any one of claims 1 to 12.

14. The method according to claim 13 wherein the nuclear donor cell has been selected on the basis that it is histone hypomethylated.

15. A method of producing a foetus, the method comprising allowing an embryo obtained by a method according to any one of claims 1 to 14 to develop into a foetus.

16. A method of producing a non-human animal the method comprising allowing an embryo obtained by a method according to any one of claims 1 to 14 or a foetus obtained by a method according to claim 15 to develop into said non-human animal.

17. A method of producing an embryonic stem cell line, the method comprising transferring an embryo obtained by the method of any one of claims 1 to 14 to a culture system.

18. A method of producing an embryonic stem cell line, the method comprising isolating the inner cell mass of an embryo obtained by the method of any one of claims 1 to 14 and transferring the inner cell mass to a culture system.

19. A method according to any one of the preceding claims wherein the nuclear donor cell is a non-human cell.

20. A method according to any one of the preceding claims wherein the recipient cell is a non-human cell.

21. An embryo obtained by the method of any one of claims 1 to 14 wherein the embryo is preferably a non-human embryo.

22. A foetus obtained by the method of claim 15, wherein the embryo is preferably a non-human foetus.

23. A non-human animal obtained by the method of claim 16.

24. An embryonic cell obtained by the method of claim 17 or 18, wherein the embryonic cell is preferably a non-human cell.

25. The use of histone hypomethylation status as an indicator of the suitability of a cell to act as a nuclear donor cell.

ABSTRACT

The present invention provides a method of producing an animal embryo, the method comprising transferring from a nuclear donor cell which has been selected on the basis that it is histone hypomethylated at least a portion of the nuclear contents including at least the minimum chromosomal material able to support development into a suitable recipient cell.

FIG. 1

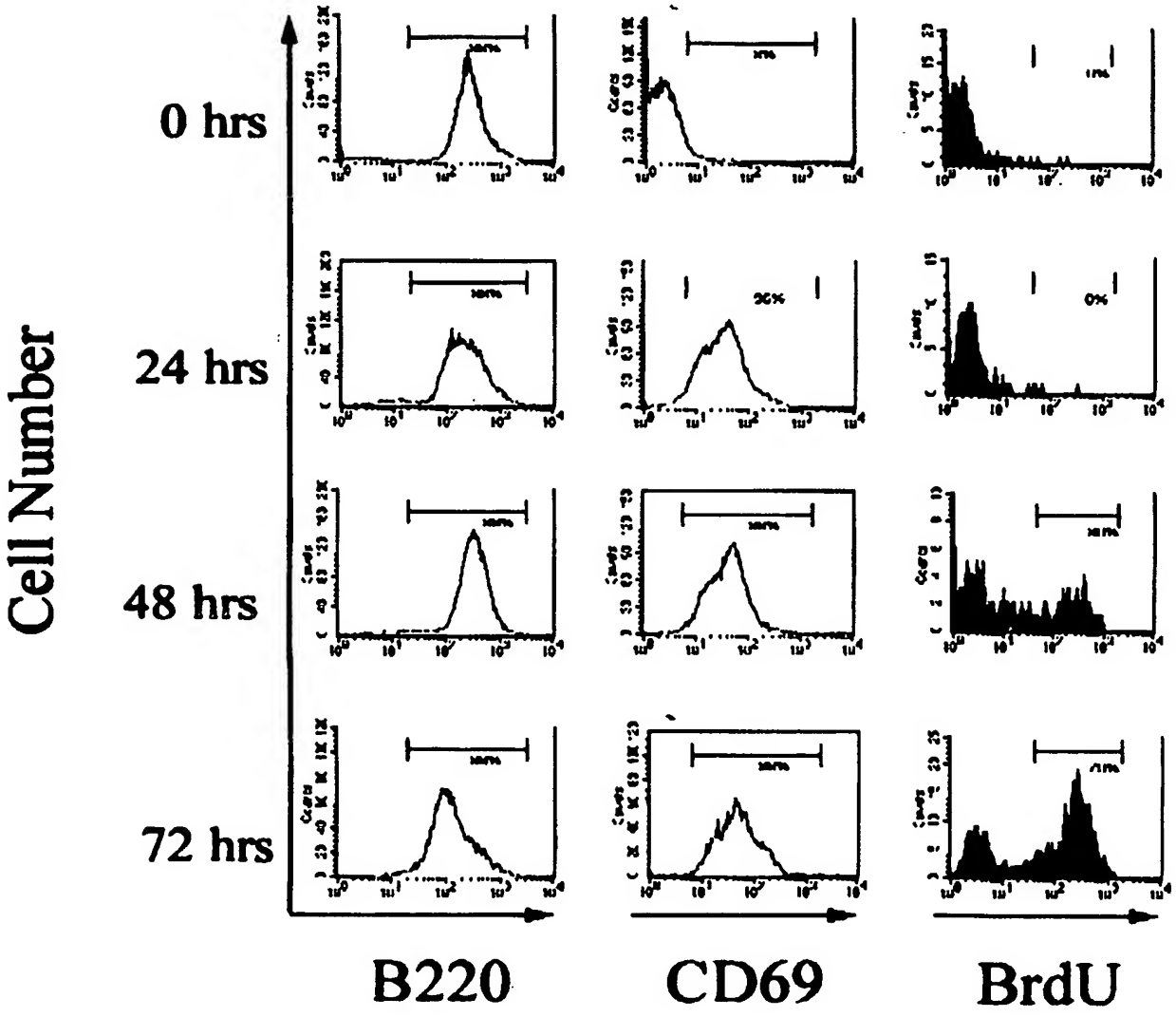


FIG. 2

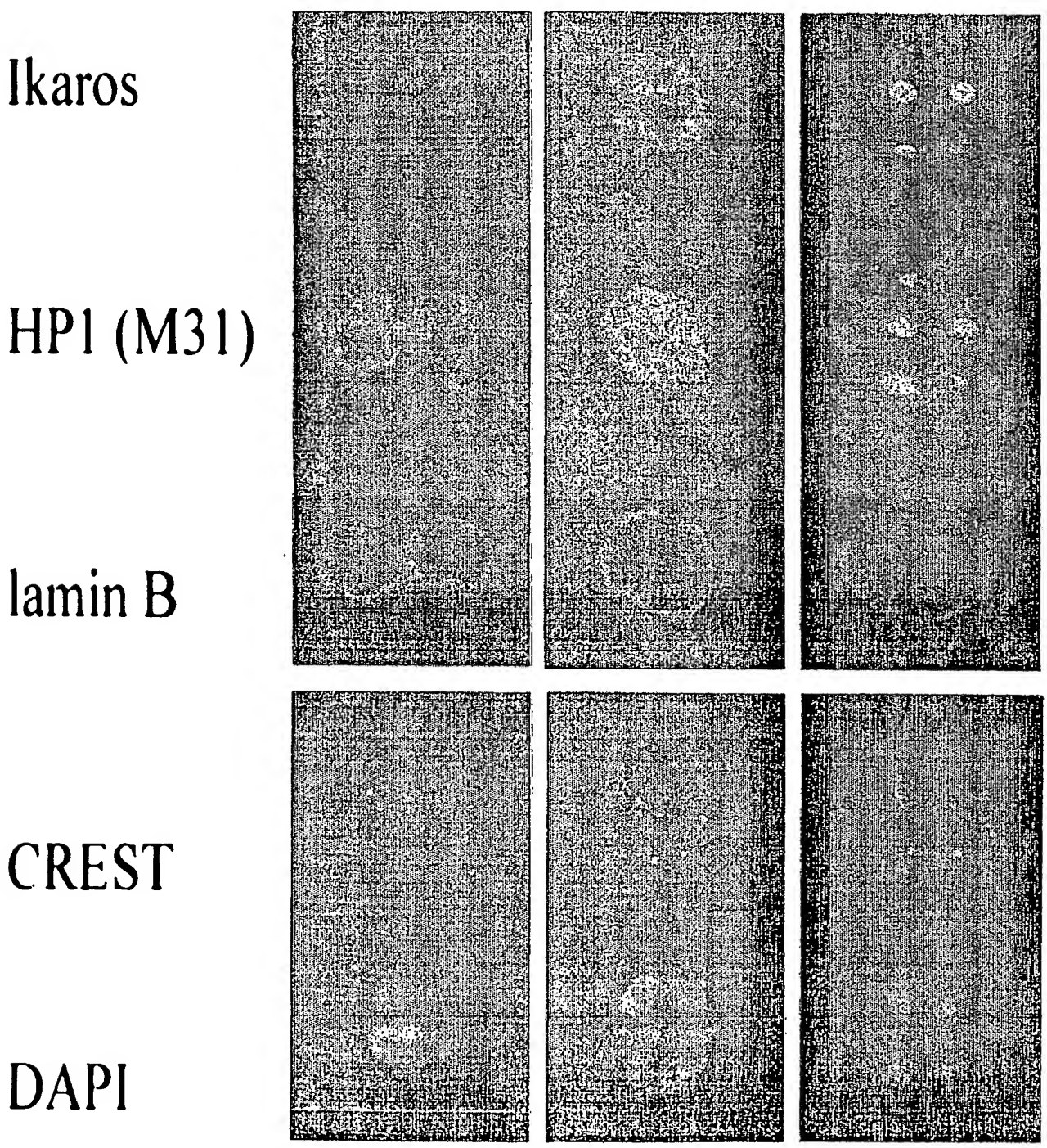


FIG. 3a

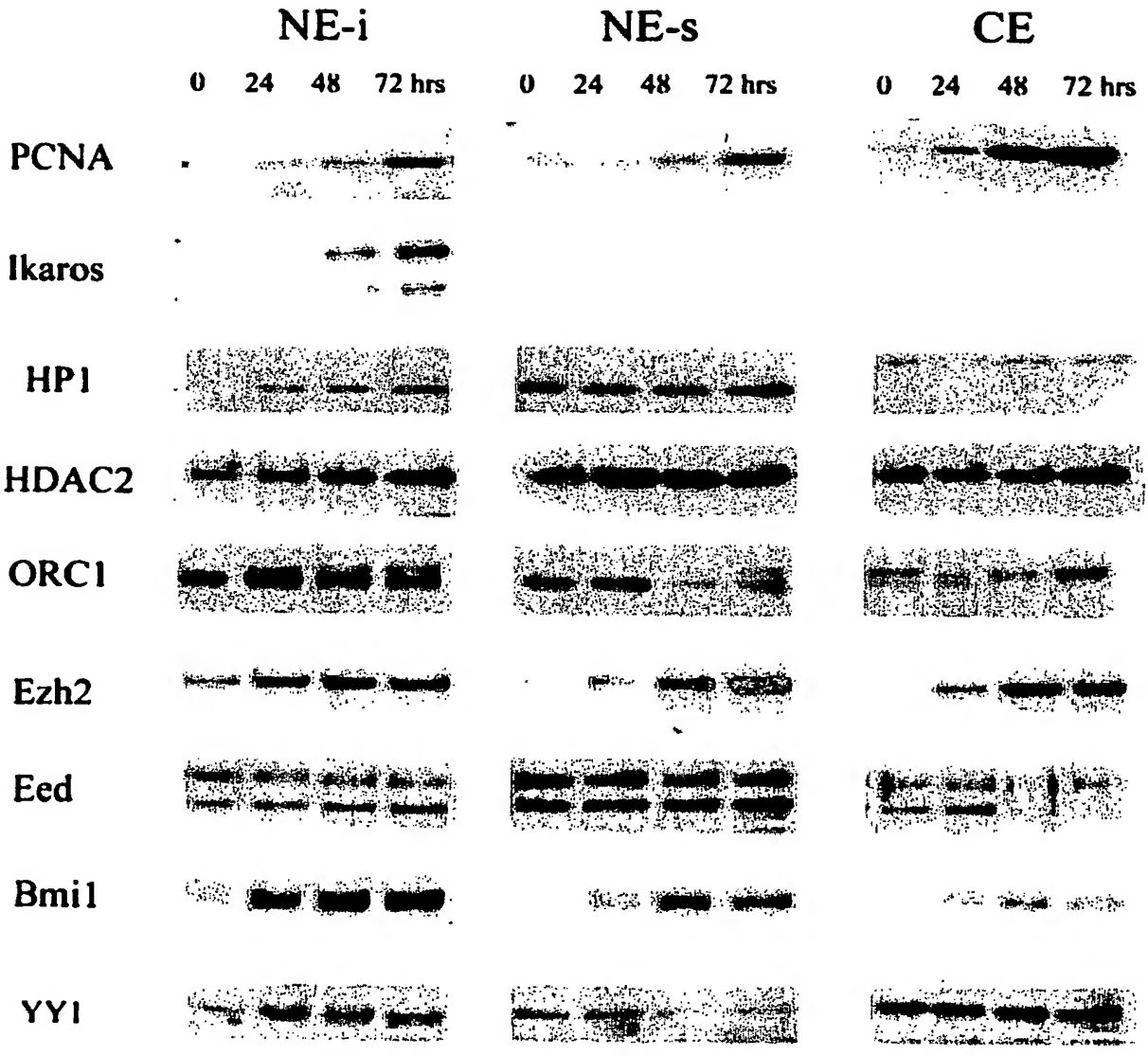

NE-i
NE-s
CE
0 24 48 72 hrs
0 24 48 72 hrs
0 24 48 72 hrs
PCNA
Ikaros
HP1
HDAC2
ORC1
Ezh2
Eed
Bmi1
YY1

FIG. 3b

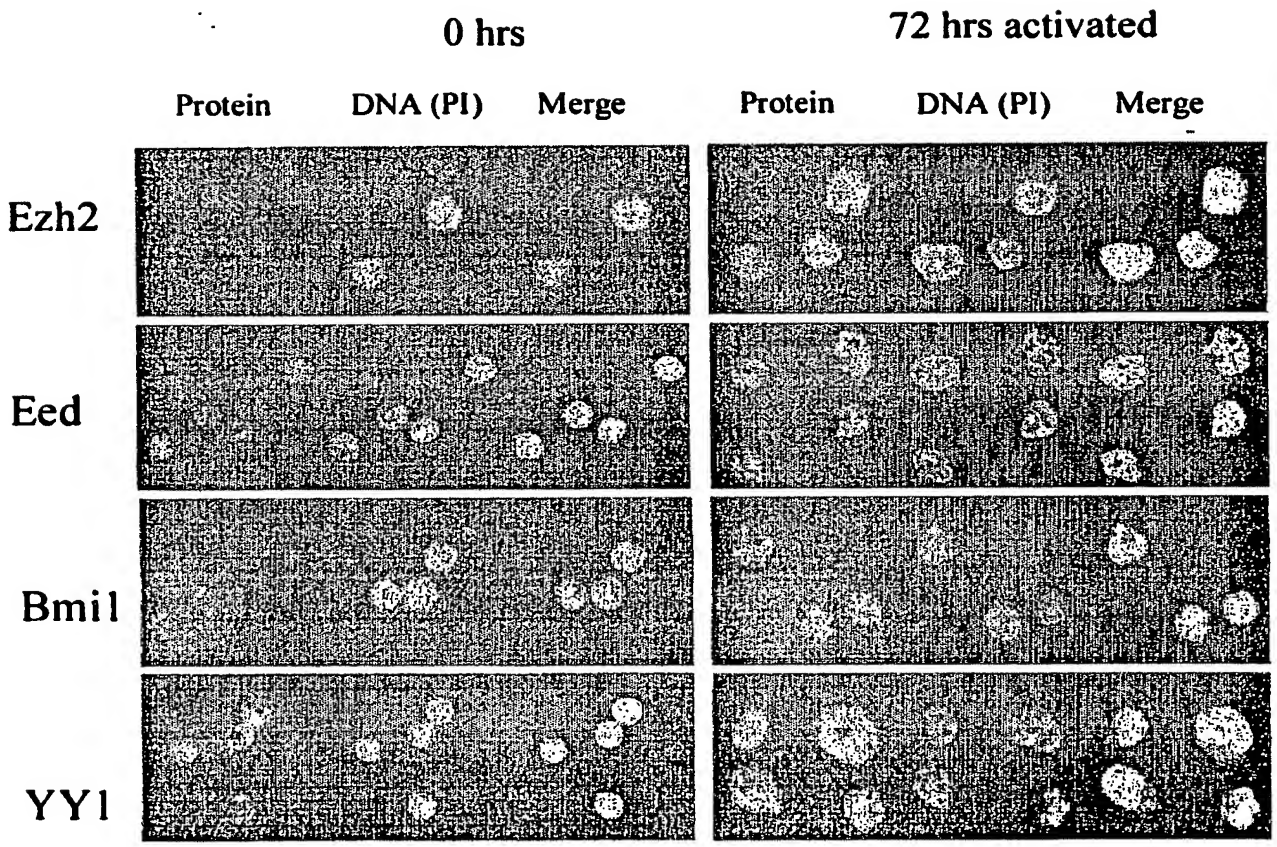

0 hrs
72 hrs activated
Protein
DNA (PI)
Merge
Protein
DNA (PI)
Merge
Ezh2
Eed
Bmi1
YY1

FIG. 4

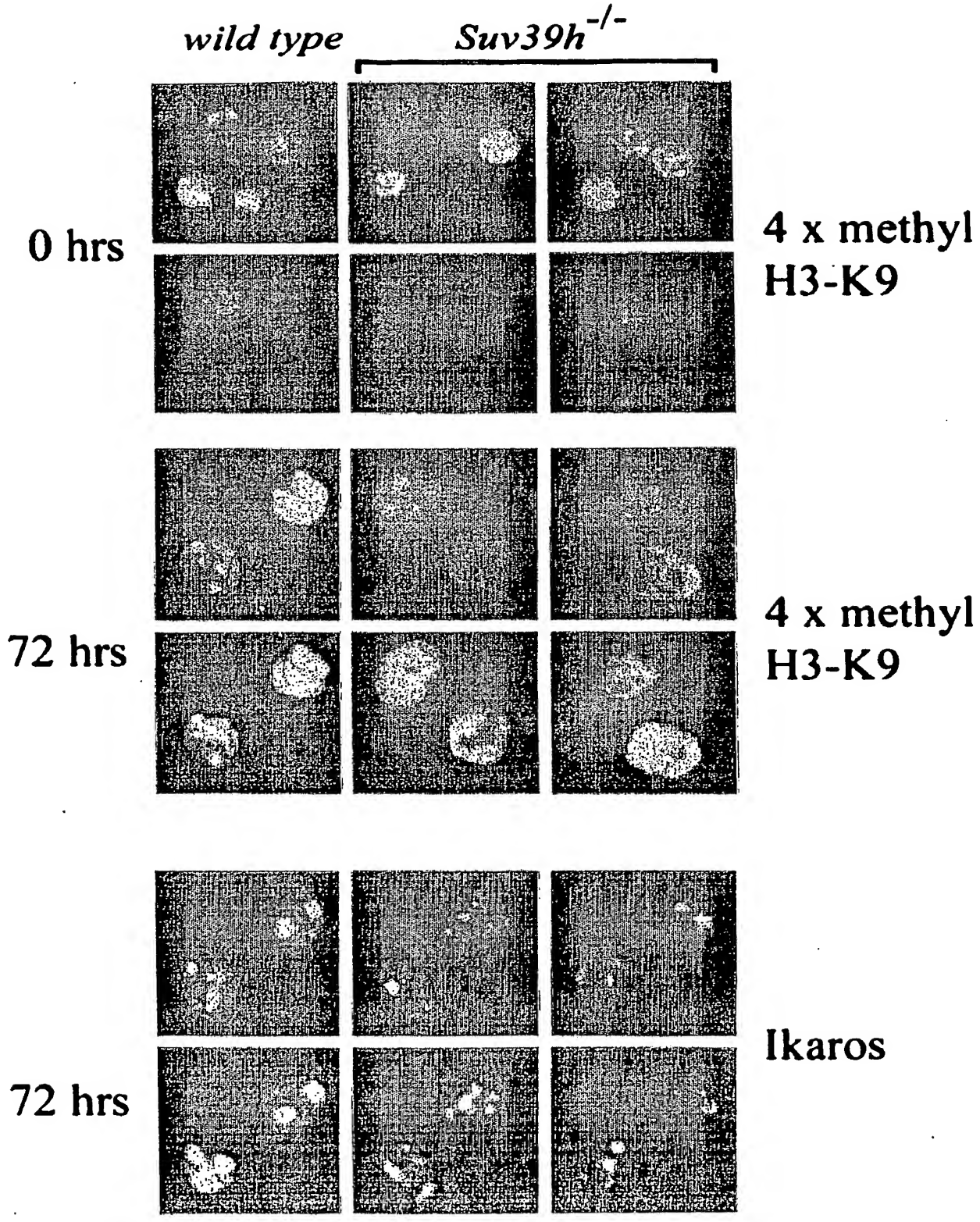

wild type
Suv39h-/-
0 hrs
4 x methyl H3-K9
72 hrs
4 x methyl H3-K9
72 hrs
Ikaros

FIG. 5a

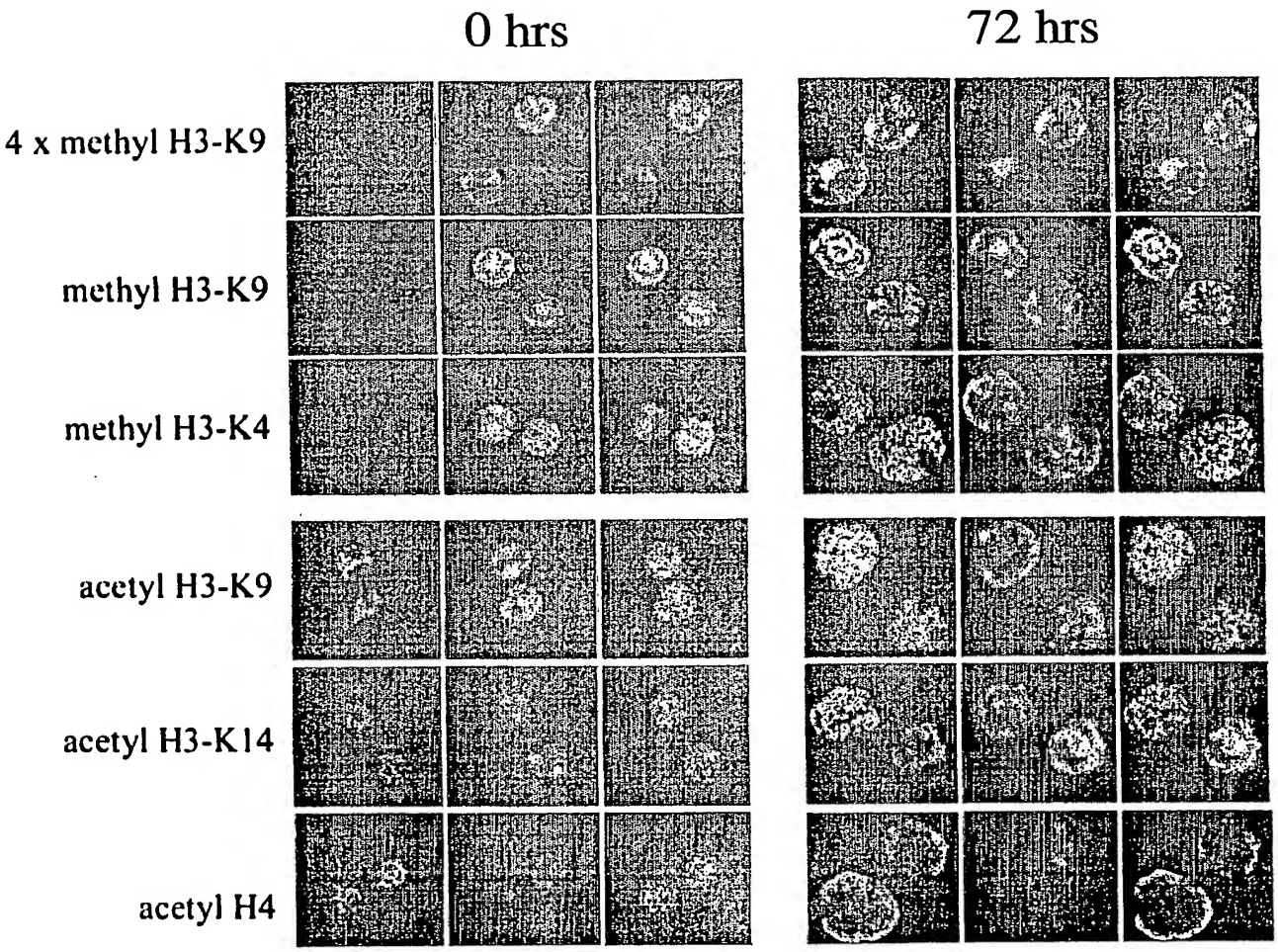
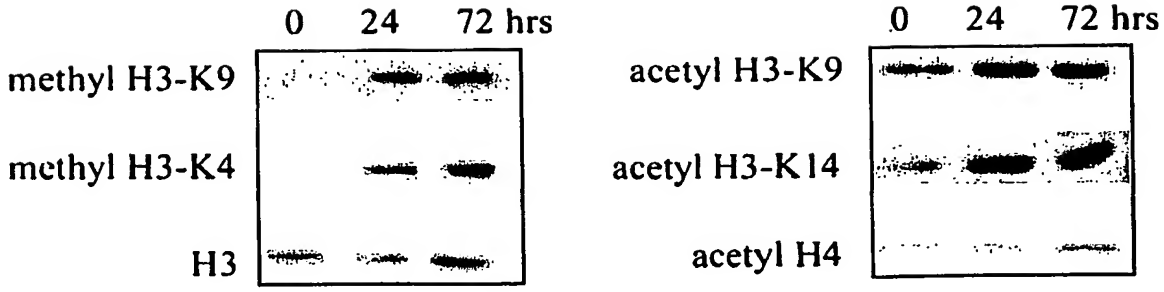
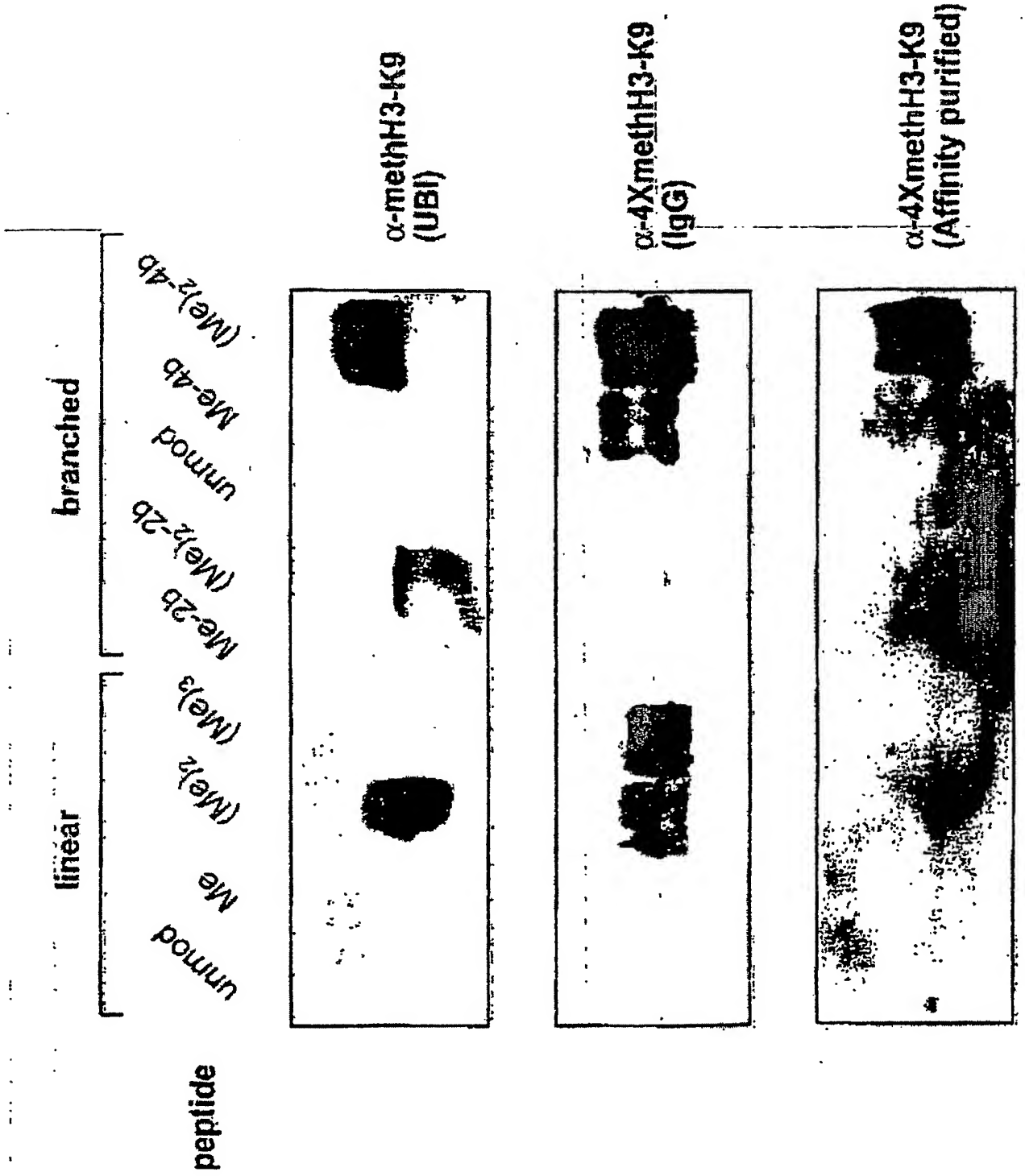

0 hrs
72 hrs
4 x methyl H3-K9
methyl H3-K9
methyl H3-K4
acetyl H3-K9
acetyl H3-K14
acetyl H4

FIG. 5b


0 24 72 hrs
methyl H3-K9
methyl H3-K4
H3
0 24 72 hrs
acetyl H3-K9
acetyl H3-K14
acetyl H4

supplementary information (i)

FIGURE 6



supplementary information (ii) FIGURE 7

Adult liver cryosections